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ORIGINAL ARTICLE

Development of a novel glycoprotein-based immunochromatographic test for the rapid serodiagnosis of bovine brucellosis

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Abstract

Aims: Bovine brucellosis is a worldwide zoonotic disease that causes important economic losses and public health concerns. Because control of the disease depends on vaccination, serodiagnosis and isolation of the infected animals, affordable, rapid and accurate point of care (POC) tests are needed.

Methods and Results: We developed and evaluated a novel glycoprotein-based immunochromatographic test for the detection of IgG antibodies against the *O*-polysaccharide of *Brucella* in bovine serum samples. *Brucella* GlycoStrip combines the power of immunochromatographic and bacterial glycoengineering technologies for the diagnosis of bovine brucellosis. The analysis of positive and negative reference samples indicated that the test has a diagnostic sensitivity and specificity of 96.9% (95% CI: 92.7%–100.0%) and 100%, respectively.

Conclusions: Due to the recombinant glycoprotein-based antigen OAg-AcrA, which consists of the *O*-side chain of *Brucella* smooth lipopolysaccharide (sLPS) covalently linked to the carrier protein AcrA, the test is highly accurate, allows the differentiation of infected animals from those vaccinated with a rough strain or with a single dose of a smooth strain and fulfil the minimum diagnostic requirements established by the national and international regulations.

Significance and Impact of Study: This strip test could provide a rapid (10 min) and accurate diagnosis of bovine brucellosis in the field contributing to the control of the disease.

KEYWORDS

bovine brucellosis, *Brucella*, diagnostics, glycoproteins, immunochromatography, lateral flow immunoassay, serodiagnosis

INTRODUCTION

Brucellosis is a highly contagious zoonotic disease caused by Gram-negative bacteria of the genus *Brucella* that affects livestock, wild animals and humans (Corbel, 1997). *Brucella abortus* is the main aetiological agent of bovine brucellosis, a disease that affects cattle productivity. The most common clinical manifestations of the infection are reproductive loss with infertility, abortion in the third trimester, placental retention, premature births of weak offspring and reduced milk production. In bulls, it can cause infertility, epididymitis and orchitis (Herrera et al., 2008; Olsen & Tatum, 2010). In addition, detection of the disease in a region or country imposes, due to international

regulations, restrictions on animal transportation and trade (Seleem et al., 2010). For the reasons stated before, bovine brucellosis results in a great economic burden for the livestock activity, highlighting the importance of monitoring herds to detect and separate the infected animals from the healthy population. Because of its zoonotic characteristics, brucellosis in humans can be severely debilitating and disabling and remains an important public health concern, particularly in endemic regions (Young, 1995). In the absence of a vaccine for humans, prevention of the disease depends primarily on the control of brucellosis in animals (the natural reservoir of the disease) (Godfroid et al., 2010). Due to these characteristics, timely and accurate diagnosis of bovine brucellosis is not only important for the correct management of the animals, avoiding the spreading of the disease and increasing the productivity, but also to prevent the infection of humans.

Control of bovine brucellosis depends primarily on vaccination in combination with the detection and removal of the infected animals. Due to the absence of pathognomonic clinical signs, diagnosis must be confirmed by laboratory tests. Isolation of *Brucella* from blood or other tissues remains the gold standard for the diagnosis of brucellosis. However, due to the slow growth of the bacterium in primary cultures (up to 7 days), the risk involved in the manipulation and the poor sensitivity, isolation by microbiological methods is not routinely used as a screening test. Instead, laboratory diagnosis of bovine brucellosis is mainly based on detecting specific antibodies against the infectious agent present in serum and/or milk samples. An essential property of these serological tests should be the capacity to differentiate infected from vaccinated animals (DIVA strategy) so that they can be applied in regions or countries that have brucellosis control programmes with vaccination (Seleem et al., 2010).

The most widely used serological tests for the diagnosis of bovine brucellosis are the agglutination tests, including the buffered plate agglutination test (BPAT), rose Bengal plate agglutination test (RBPT), serum agglutination test (SAT) and milk ring test (MRT), and the complement fixation test (CFT) (DILAB-SENASA, 2009; OIE, 2012). Indirect enzyme-linked immunosorbent assays (iELISAs) have also been developed (Gall & Nielsen, 2004). All these assays use complex mixtures of antigens like whole inactivated bacteria, bacterial extracts enriched in smooth lipopolysaccharide (sLPS) or purified sLPS. For this reason, they suffer from a high rate of false-positive results due to cross-reactivity against common antigens and epitopes, especially those present in the lipid A-core section of LPS shared by *Brucella* and other Gram-negative bacteria. In addition, these tests do not clearly differentiate infected from vaccinated animals due to the antigenic similarity of the strain used to formulate the vaccine with respect to the virulent strains. An exception to these tests are the competitive ELISA (cELISA) and the fluorescence polarization assay (FPA) which specifically detect anti-*O* polysaccharide antibodies (McGiven et al., 2003; Nielsen, 1990; Nielsen et al., 1995; Nielsen et al., 1996).

We have previously developed a recombinant glycoprotein-based antigen (OAg-AcrA) for the diagnosis of brucellosis in which the *O* polysaccharide section of the LPS of *Brucella* (OAg) is covalently linked to the carrier protein AcrA (Iwashkiw et al., 2012). OAg-AcrA was produced by bacterial glycoengineering (Wacker et al., 2002), a technology that combines bacterial glycobiology with genetic engineering to obtain glycosylated recombinant proteins using non-pathogenic bacteria as hosts. These recombinant glycoproteins have been used to develop glycoconjugate vaccines, protein drugs and diagnostics (Cuccui & Wren, 2015; Harding & Feldman, 2019; Melli et al., 2015). To produce the OAg-AcrA antigen, the oligosaccharyltransferase (OTase)-dependent N-glycosylation pathway is exploited (Feldman et al., 2005). In this system, the *O* polysaccharide is transferred by the oligosaccharyltransferase PglB from the lipid carrier undecaprenol to the carrier protein AcrA in the bacterial periplasm, resulting in the synthesis of the OAg-AcrA (Iwashkiw et al., 2012). Coupling OAg-AcrA to magnetic beads or ELISA plates and using different detection systems (spectrophotometric, electrochemical or fluorescent detection) (Cortina et al., 2016b), as well as different types of samples (serum, whole blood and milk), allowed us to develop different indirect immunoassays for the diagnosis of human (Ciocchini et al., 2013), bovine (Ciocchini et al., 2014) and porcine brucellosis (Cortina et al., 2016a), demonstrating the usefulness of OAg-AcrA for the diagnosis of brucellosis caused by smooth *Brucella* strains (*B. abortus*, *B. melitensis* and *B. suis)*.

In this work, we have developed a novel one-step immunochromatographic assay for the detection of specific antibodies against the *O* polysaccharide of *Brucella* in bovine serum samples. *Brucella* GlycoStrip combines the power of the immunochromatographic with the glycoengineering technology for the rapid (10 min) and accurate diagnosis of bovine brucellosis. The use of the recombinant glycoprotein OAg-AcrA results in a test with a diagnostic sensitivity and specificity of 96.9% and 100%, respectively, that additionally allows the differentiation of naturally infected animals from those vaccinated with a rough strain or with a single dose of a smooth strain.

MATERIALS AND METHODS

Expression and purification of the antigen (OAg-AcrA)

Expression and purification of the *O*-polysaccharide–protein conjugate (OAg-AcrA recombinant glycoprotein) was

performed as previously described (Ciocchini et al., 2013; Iwashkiw et al., 2012). *Yersinia enterocolitica* O:9 wild-type strain transformed with the plasmids pMAF10 (encoding the *Campylobacter jejuni* oligosaccharyltransferase OTase PglB) and pMH5 (encoding the *C. jejuni* carrier protein AcrA fused to a histidine tag) was grown on Luria-Bertani medium (LB; 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) at 37°C for 2.5 h (OD_{600} ~0.5) and PglBCj expression was induced with arabinose 0.2% (w/v). Four hours after the first induction, PglBCj was re-induced by a second addition of arabinose to maximize AcrA glycosylation. After 20 h of induction, the periplasmic extract was obtained by a two-step osmotic shock method (Caillava et al., 2019) and the OAg-AcrA was purified by metal-chelate affinity chromatography.

Sera

Three different sample panels were analysed. Panel I. Serum samples obtained from four groups of animals were included in a controlled vaccination/challenge trial performed previously by our laboratory (Table S1). Group 1 included non-vaccinated pregnant heifers experimentally infected with the wild-type strain *B. abortus* 2308 (nine animals, 41 samples). The serum samples were obtained before the infection, on the day of infection and at different times post-infection. Group 2 consisted of non-infected/non-vaccinated animals (eight animals, 8 samples). Group 3 included S19-vaccinated pregnant heifers challenged with *B. abortus* 2308 (nine animals, 54 samples). Serum samples were obtained before vaccination, at different times post-vaccination and post-challenge. Group 4 included pregnant heifers vaccinated twice 16 months apart with the rough vaccine strain *B. abortus* Δ*pgm* and challenged with *B. abortus* 2308 (five animals, 30 samples). Serum samples were obtained pre-vaccination, at different times post first and second vaccination and post-challenge with the wild-type strain. Due to the clean deletion of the phosphoglucomutase gene (*pgm*), *B. abortus* Δ*pgm* is unable to assemble the *O* polysaccharide to the lipid A-core, resulting in a rough phenotype strain containing an incomplete LPS (Ugalde et al., 2000; Ugalde et al., 2003). Panel II. Serum samples obtained from naturally infected and non-infected/S19-vaccinated cattle were provided by the National Brucellosis Reference Laboratory (DILAB-SENASA), Argentina (Table S2). *Negative reference samples*: 216 serum samples obtained from S19-vaccinated animals were from officially certified brucellosis-free herds. These samples were obtained from 6 months after vaccination with a single dose of the smooth vaccine strain S19 and were serologically negative by the standard screening test BPAT (n = 159) or were BPAT-positive but negative by cELISA, FPA or CFT ($n = 57$). *Positive reference samples*: 65 serum samples obtained from animals of brucellosis-positive herds. These samples were positive by BPAT and confirmed by at least two positive results by CFT, cELISA and/or FPA. Panel III. 402 encoded serum samples obtained from two different livestock establishments located in the province of Córdoba, Argentina, and included in the double-blind study (Table S3).

Control sera

-*Positive- and negative-control sera*. The positive-control serum (POS) was obtained from a non-vaccinated animal experimentally infected with the wild-type strain *B. abortus* 2308 (serological tests results: BPAT, positive; cELISA, 100%; FPA, 287 mP; CFT, 1/8++; VETLIS® *Brucella* Glyco-iELISA Bovine, 100%). The negative-control serum (NEG) was obtained from a non-infected/non-vaccinated animal (serological tests results: BPAT, negative; cELISA, 21%; FPA, 49 mP; CFT, negative; VETLIS® *Brucella* Glyco-iELISA Bovine, 5%) coming from a certified free-brucellosis herd. These controls were used to evaluate each new batch of strips.

-*SENASA_OIE standard sera*. Strong positive ($SENASA_{SP}SS$), weak positive ($SENASA_{WP}SS$) and negative ($SENASA_{NEG}SS$) reference standard provided by DILAB-SENASA, Argentina.

Ethics statement

In this study, we have analysed a characterized sera collection provided by the DILAB-SENASA and serum samples obtained from animals were included in a controlled vaccination/challenge trial performed previously by our laboratory. No animals were used in the course of this work.

Brucella GlycoStrip assay development and optimization

The structure of the *Brucella* glycostrips and its principle are shown in Figure 1A. For each strip, the conjugate pad (CP), nitrocellulose membrane (M), absorbent pad (W) and cover tapes (CT) were laminated over a plastic backing card (BC) coated with a pressure sensitive adhesive. Colloidal gold (Au)-labelled goat anti-bovine IgG antibodies were dispensed into the CP and the M was stripped with the OAg-AcrA antigen and donkey anti-goat IgG antibodies at the test line (TL) and control line (CL), respectively. After dispensing, the M was blocked and dried, and all the materials were laminated over the BC. Afterwards,

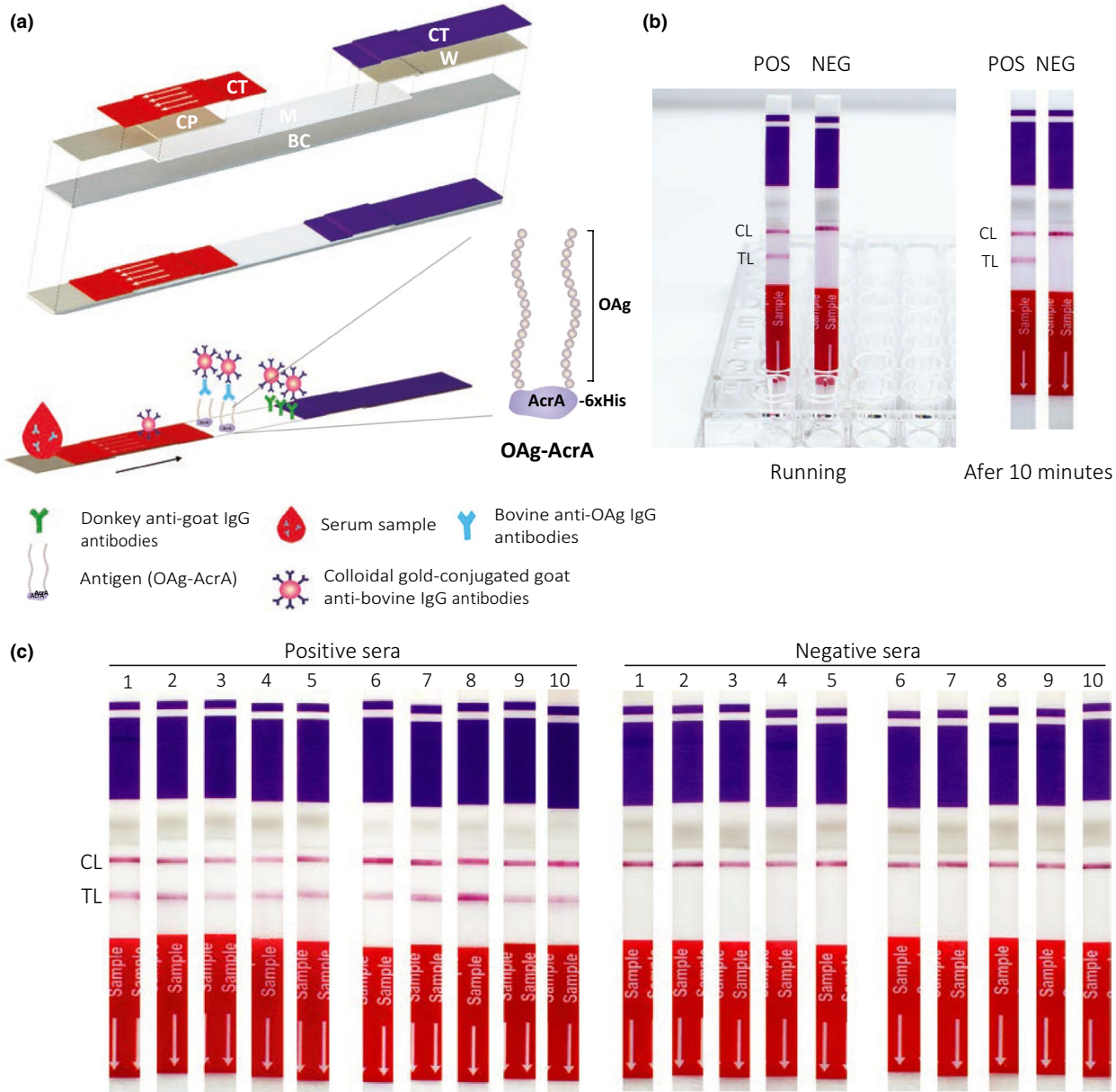

FIGURE 1 *Brucella* GlycoStrip. (A) Schematic diagram of the strip structure in dipstick format and principle of the assay. The device consists of a backing card (BC) into which the conjugate pad (CP), nitrocellulose membrane (M), absorbent pad (wick, W) and cover tapes (CT) are laminated. Colloidal gold (Au)-labelled goat anti-bovine IgG antibodies are dispensed into the CP and the nitrocellulose membrane is stripped with the OAg-AcrA antigen and donkey anti-goat IgG antibodies at the test line (TL) and control line (CL), respectively. The arrow indicates the direction of liquid flow during the assay. The sample migrates to the CP where re-mobilizes the dried colloidal gold conjugate. Bovine IgGs react with the Au-labelled goat anti-bovine IgG antibodies to form complexes that migrate into the membrane; antibody-Au complexes that contain specific IgG antibodies against the *Brucella* OAg are captured at the TL and the antibody-Au complexes not retained at the TL are captured at the CL. Excess reagents are absorbed by the W. (B) Pictures showing lateral flow strips in dipstick format for the positive and negative control sera (PC and NC) running and after 10 min. The assay is performed by placing the strip into a test tube or flat-bottom microplate containing the running buffer and 5 µl of serum. The result of the test is read after 10 min by visual inspection for staining of the TL and CL. No reaction at the CL invalidates the test; the control line should stain in all cases. The assay is scored negative when no staining of the TL occurred and positive when a distinct staining of the TL is observed. The TL may stain at different intensities depending on the titre of specific antibodies in the sample. POS, positive-control serum; NEG, negative-control serum. (C) Pictures of strips in dipstick format showing the results for 10 representative positive and negative samples of the panel used to evaluate the assay

two self-adhesive cover tapes were placed manually over the laminated sheet: one in the upper part of the sheet covering the W and the overlap of the W with the M, and the other in the lower part covering the overlap of the CP with the M. The cover tapes protect the strip, indicate how the strip should be handled and placed in the test tube and

ensure the correct overlapping of the different materials. Finally, laminated sheets were cut into strips and stored in a moisture-resistant pouch with a silica gel packet inside. Optimization of the conjugate, antigen, and antibody concentration and the selection of the different materials were performed in a step-by-step procedure analysing a panel of control positive and negative serum samples. The assay was performed by placing the strip into a Khan test tube containing the running buffer and 5 µl of serum sample. The results were read at 10 min by visual inspection for staining of the TL and CL. The CL should stain in all cases; no staining of the CL invalidates the test. The assay was scored negative when no staining of the TL occurred and positive when distinct staining of the TL was observed.

Brucella GlycoStrip assay is based on the immunological capture of gold nanoparticles coated with goat anti-bovine IgG antibodies during its passage through the M. The IgG isotype immunoglobulins present in the serum sample react with the gold particles functionalized with the anti-bovine IgG antibodies and the complexes migrate by chromatography to the reaction zone in the M. If the sample contains anti-*Brucella O* polysaccharide IgG antibodies, the nanoparticle–antibody complexes will be captured by the antigen immobilized at the TL which will be visualized as a red/purple line (Figure 1B,C). Whether the sample contains IgG anti-*Brucella O* polysaccharide antibodies or not, the complexes that are not captured at the TL continue to migrate and are captured at the CL by the anti-goat IgG antibodies, also leading to the formation of a red/purple line. The appearance of the CL indicates that the chromatography has been developed correctly and under conditions that ensure the antigen–antibody reactions.

Data analysis

The diagnostic sensitivity (Se) was calculated by performing the test for 65 positive reference samples and using the following formula: Se = [(number of true positives) / (number of true positives) + (number of false negatives)] x 100. Diagnostic specificity (Sp) was calculated from 216 negative reference samples and using the formula Sp = [(number of true negatives) / (number of true negatives + number of false positives)] x 100. Positive predictive value (PPV) was calculated with the formula PPV = [(number of true positives) / (number of true positives) + (number of false positives)] x 100 and the negative predictive value (NPV) with the formula NPV = [(number of true negatives) / (number of true negatives + number of false negatives)] x 100. The TL/CL ratio was calculated by a densitometric analysis of the strip signals using the Fiji-ImageJ software (Schindelin et al., 2012). All the strips were scanned using the same settings and the signal intensity was determined for the TL and CL. For each strip, the signal intensity of the zone between the TL and CL (background signal) was subtracted from the signal intensity of the TL and CL, and the TL/CL ratio was calculated.

Other tests

BPAT, SAT, SAT 2-mercaptoethanol (SAT-2ME), FPA, cELISA and CFT were performed by the National Brucellosis Reference Laboratory (DILAB-SENASA), Argentina, as previously described (DILAB-SENASA, 2009; OIE, 2012). For the BPAT, any degree of agglutination was considered positive and for FPA a reactivity value ≤94 millipolarization units (mP) was considered negative, between 95 and 105 mP indeterminate and > 105 mP positive. For the CFT, a reactive value ≥36 UI/ml (dilution 1:8 +) was considered a positive result. For the cELISA, a percentage of inhibition ≥40% was considered positive. The VETLIS® *Brucella* Glyco-iELISA Bovine (Chemtest Argentina S. A.) test was performed according to the manufacturers' instructions. This assay is an iELISA based exclusively on the detection of *Brucella* anti-*O* polysaccharide IgG antibodies in bovine serum or milk samples that uses the recombinant glycoprotein OAg-AcrA as antigen (Ciocchini et al., 2014). For this test, a reactivity value <11% was considered negative, ≥ 11% ≤ 20% indeterminate and > 20% positive.

Statistical analysis

Sensitivity and specificity were estimated by observed proportions based on each reference standard, with 95% confidence intervals calculated using the simple asymptotic method. Inter-rater agreement for qualitative (categorical) items was measured by Cohen's kappa statistic (κ) (Viera & Garrett, 2005).

RESULTS

Brucella GlycoStrip development and diagnostic performance evaluation

A recombinant glycoprotein-based immunochromatographic assay in dipstick format (*Brucella* GlycoStrip) was developed as indicated in Materials and Methods. *Brucella* GlycoStrip is a one-step strip test for the detection of specific IgG antibodies against the *O* polysaccharide of the sLPS of *B. abortus*, *B. melitensis* and *B. suis* in serum samples. To assess the usefulness of the *Brucella* GlycoStrip

for the diagnosis of bovine brucellosis, a panel of serum samples obtained from four groups of animals included in a controlled vaccination/challenge trial performed previously in our laboratory was analysed (Figure 2 and Table S1). The first group included animals experimentally infected with the wild-type strain *B. abortus* 2308. Serum samples were obtained at 60 days before the infection, the day of the infection and 21, 33 and 180 days post-infection (dpi). In seven of nine animals, specific IgG antibodies were detected as early as 21 dpi and, in all animals, an increasing reactivity was observed at 33 and 180 dpi (Figure 2A). As expected, no reactivity was observed with the serum samples obtained before the infection and from the non-infected/non-vaccinated animals (Group 2, Figure 2B). These results indicate that with the *Brucella* GlycoStrip it is possible to differentiate infected from non-infected animals. Additionally, these results correlated very well with those obtained with the BPAT screening test and the commercial kit VETLIS® *Brucella* Glyco-iELISA Bovine (Chemtest Argentina S. A.) which also uses the recombinant glycoprotein OAg-AcrA as antigen (Table S1). To determine the ability of the *Brucella* GlycoStrip to differentiate infected from vaccinated animals, we analysed serum samples obtained from experimentally vaccinated animals that were challenged with *B. abortus* 2308 at the seventh month of pregnancy (Groups 3 and 4). Group 3 included animals that were vaccinated with the smooth vaccine strain S19, one of the most widely used vaccines for cattle vaccination. As a smooth strain, S19 expresses the *O*-side chain on its LPS which induces a serological response against this molecule. When calves are vaccinated between 3 and 6 months of age with a single subcutaneous dose, anti *O* polysaccharide antibody titres become negative between 6 and 9 months post-vaccination. In this group, six samples were analysed for each animal including a pre-vaccination sample, four samples obtained at 30, 395, 520 and 630 days post-vaccination (dpv) and one sample obtained at 21 days post-challenge (dpc) with *B. abortus* 2308. For this group, positive results were obtained at 30 dpv, which became negative for the rest of the post-vaccination times analysed. As expected, samples obtained at 21 dpc were positive (Figure 2C). These results correlated perfectly with those obtained by BPAT and Glyco-iELISA (Table S1). Finally, we analysed the serum samples obtained from animals that were twice vaccinated with the rough vaccine strain *B. abortus* Δ*pgm* (Group 4), a live attenuated strain lacking the phosphoglucomutase gene (pgm) (Ugalde et al., 2003). This strain can synthesize the *O*-side chain on the lipid intermediary but is unable to transfer it to the lipid A-core, resulting in a rough phenotype strain containing an incomplete LPS (Ugalde et al., 2000). In this group, six samples were analysed for each animal including a pre-vaccination sample, two samples obtained at 30- and 395-day post first vaccination, two samples obtained at 30 and 150 days post second dose of vaccine and one sample obtained at 21 dpc. Unlike the group vaccinated with S19, no reactivity was observed either after the first dose of vaccine (30 dpv and 395dpv) or in the rest of the samples taken after the second dose (30 dp2v and 150 dp2v) (Figure 2D). As expected, a strong seroconversion was detected in all the samples analysed at 21 days post-challenge with the virulent smooth strain. (Figure 2D). It is noteworthy that 3 of the 5 samples obtained 30 days after the second dose were positive by BPAT (Table S1). This is not surprising since the BPAT test uses the whole bacterium as antigen. The lack of reactivity observed after the first and the second dose is consistent with the rough phenotype of the *B. abortus Δpgm* vaccine strain since both *Brucella* GlycoStrip and Glyco-iELISA specifically detect antibodies against the O-side chain of *Brucella* sLPS.

To evaluate the diagnostic specificity and sensitivity of *Brucella* GlycoStrip, serum samples obtained from confirmed brucellosis-positive (positive reference samples, n = 65) and non-infected/S19-vaccinated (negative reference samples, n = 216) animals were tested (Table 1 and Table S2). The negative reference samples were obtained at least 6 months after vaccination with a single dose of the smooth vaccine strain S19 and not only included samples that were serologically negative by the standard screening test BPAT (n = 159) but also samples that were BPAT positive but negative by cELISA, FPA and CFT ($n = 57$) (Table S2). Of the 65 positive samples, 63 were positive with the *Brucella* GlycoStrip giving a diagnostic sensitivity of 96.9%. All the negative reference

FIGURE 2 *Brucella* GlycoStrip analysis of serum samples obtained from animals included in a controlled vaccination/challenge trial. (A) Analysis of serum samples obtained from nine animals infected with the wild-type strain *B. abortus* 2308. Serum samples obtained 60 days prior to the infection (a), the day of infection (b) and at different times post-infection (c–e) were analysed. Pictures of the strips for each animal are also shown. (B) Analysis of serum samples obtained from eight non-infected/non-vaccinated animals. Pictures of the strips showing the results obtained with these samples. (C) Analysis of serum samples obtained from nine S19-vaccinated animals and challenged with the wild-type strain *B. abortus* 2308. Serum samples obtained prior to vaccination (a), at different times post-vaccination (b–e) and 21 days post-challenge (f) were analysed. (D) Analysis of serum samples obtained from five animals vaccinated with the rough strain *B. abortus* Δ*pgm* and challenged with *B. abortus* 2308. Serum samples obtained prior to vaccination (a), at different times post first (b and c) and second vaccination (d and e), and 21 days post-challenge (f) were analysed. In bar graphs, results are expressed as the relative intensity of the test line (TL) with respect to the control line (CL) for each strip (see Materials and Methods)

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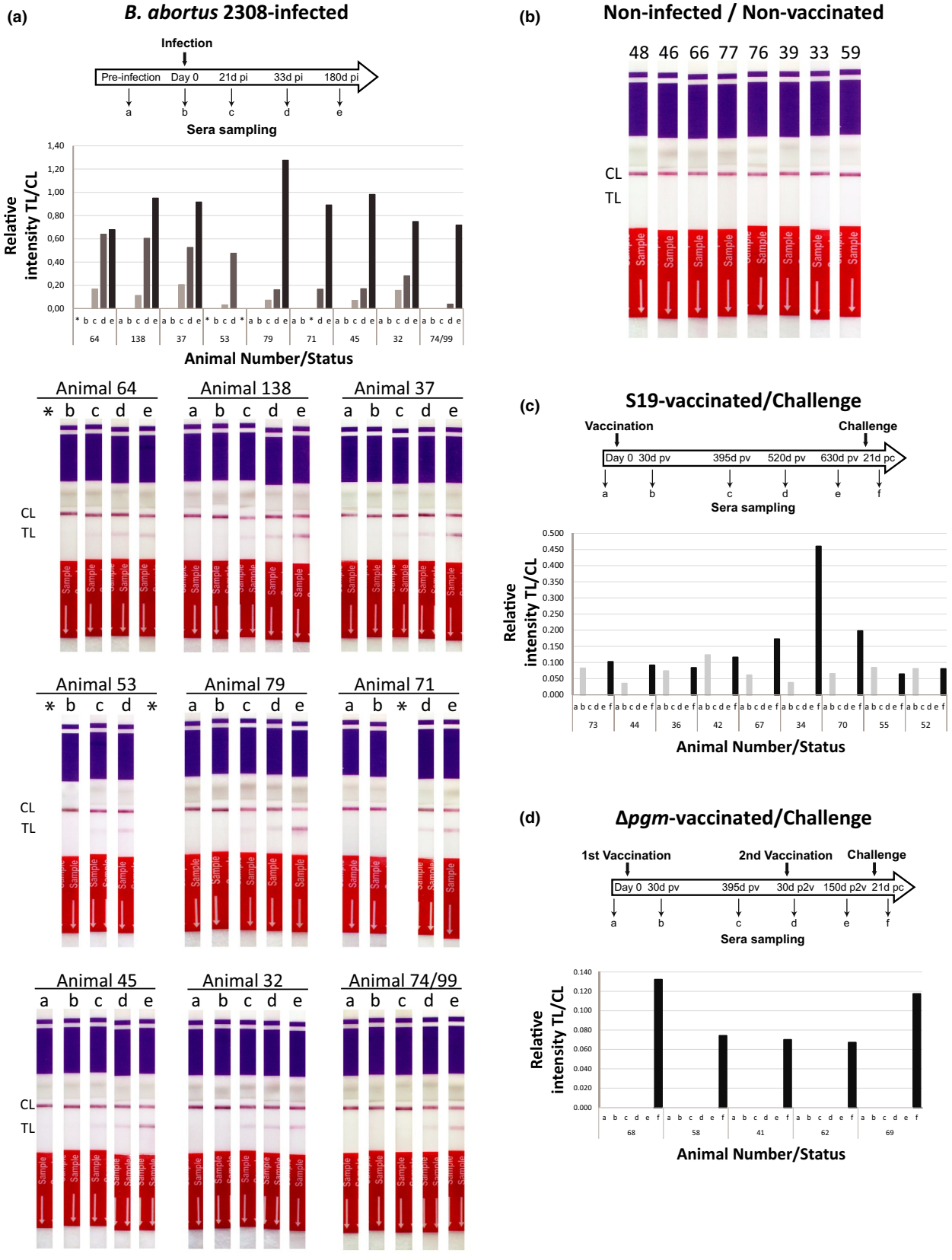

samples, including those BPAT positive, were negative with the GlycoStrip giving a diagnostic specificity of 100% (Table 1 and Table S2). Pictures of strips showing the results for 10 representative positive and negative samples of the panel used to evaluate the diagnostic performance of the assay are shown in Figure 1C. Based on these results, the calculated positive and negative predictive values were 100 and 99.1%, respectively (Table 1).

TABLE 1 *Brucella* GlycoStrip analysis of serum samples obtained from naturally infected and non-infected/S19-vaccinated cattle

		Reference samples[a]		
		POS	**NEG**	
Brucella GlycoStrip outcome	POS	63 (TP)	0 (FP)	**PPV = 100%**
	NEG	2 (FN)	216 (TN)	**NPV = 99.1%** (95%CI: 97.9–100.0%)
		Sensitivity = 96.9% (95%CI: 92.7–100.0%)	**Specificity = 100%**	

[a]Positive reference samples were confirmed by BPAT and at least two positive results by CFT, cELISA and/or FPA. Negative reference samples were serologically negative by the standard screening test BPAT or were BPAT positive, but negative by cELISA, FPA or CFT. FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive.

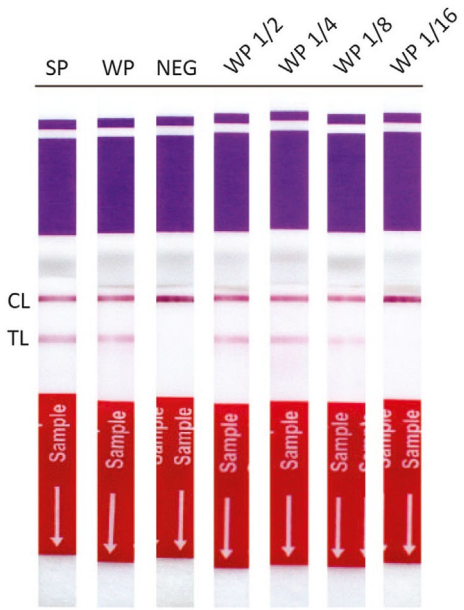

FIGURE 3 *Brucella* GlycoStrip analysis of standard sera. Pictures of strips in dipstick format showing the results for the strong positive (SP), weak positive (WP) and negative (NEG) standard sera provided by DILAB-SENASA, Argentina. $SENASA_{WP}SS$ dilutions were performed in negative serum. CL, control line; TL, test line

Finally, the strong positive ($SENASA_{SP}SS$), weak positive ($SENASA_{WP}SS$) and negative ($SENASA_{NEG}SS$) reference standards provided by the National Brucellosis Reference Laboratory (DILAB-SENASA) from Argentina were analysed. In addition, one-half serial dilutions in negative serum of $SENASA_{WP}SS$ were analysed. These standards have been established by the DILAB-SENASA laboratory, in comparison with the OIE international reference standards, to set the minimum diagnostic requirements for commercially available tests. As shown in Figure 3 and Table 2, the $SENASA_{SP}SS$ and the $SENASA_{WP}SS$ up to the dilution 1/8 showed positive results when the *Brucella* GlycoStrip was used. As expected, the $SENASA_{NEG}SS$ gave a negative result. These results are in complete agreement with those obtained with the CFT for which a reactive value ≥36 UI/ml (dilution 1:8 +) is considered a positive result and the VETLIS® *Brucella* Glyco-iELISA Bovine. For this latter test, the $SENASA_{WP}SS$ serum in dilution 1/16 was also reactive.

Taken together, these results demonstrate that the *Brucella* GlycoStrip is a highly accurate, rapid and simple serological test for the diagnosis of bovine brucellosis. The test is suitable for the use in animals vaccinated either with the rough strain *B. abortus Δpgm* or with the smooth vaccine S19 when the samples are obtained after 6 months of vaccination with a single dose. Additionally, the test has been successfully evaluated with the OIE_SENASA reference sera, overcoming the minimum diagnostic requirements established by national and international regulations.

Double-blind analysis

To further evaluate the performance of the *Brucella* GlycoStrip and compare it with a serological test that uses the same antigen but in a different diagnostic platform, a double-blind study was carried out analysing a panel of 402 encoded serum samples obtained from two livestock establishments in Argentina. All the samples were analysed blindly and in parallel by the *Brucella* GlycoStrip and VETLIS® *Brucella* Glyco-iELISA Bovine (Chemtest Argentina S. A.) assays. Both use the recombinant glycoprotein OAg-AcrA as the antigen and therefore exclusively detect anti-*Brucella* O-side chain IgG antibodies in bovine serum samples. Of the 126 positive samples detected by the Glyco-iELISA, 124 samples were also positive by GlycoStrip (Table 3 and Table S3). Samples 382 and 402 were Glyco-iELISA positive but negative by GlycoStrip (Table S3). Only one of the 35 samples with an indeterminate result by the Glyco-iELISA (Table S3, sample 156) resulted positive by the strip test. The 241 samples negative by Glyco-iELISA were also negative by the *Brucella* GlycoStrip (Table 3 and Table S3). Considering only the samples that were positive or negative by Glyco-iELISA, Cohen's statistical value (kappa) was 0.987, indicating a near-perfect correlation between Glyco-iELISA and *Brucella* GlycoStrip.

TABLE 2 *Brucella* GlycoStrip analysis of standard sera

Standard[a]	CFT results (IU/ml)[b]	CFT results (dilution)[c]	VETLIS® *Brucella* Glyco-iELISA bovine[d]	*Brucella* GlycoStrip (TL/CL)[e]
$SENASA_{SP}SS$	1323	1:256 ++	156.4	0.96
$SENASA_{WP}SS$	288	1:64 +	129.7	0.64
$SENASA_{WP}SS$ Dilution 1/2	144	1:32 +	120.6	0.53
$SENASA_{WP}SS$ Dilution 1/4	72	1:16 +	111.7	0.35
$SENASA_{WP}SS$ Dilution 1/8	36	1:8 +	77.2	0.18
$SENASA_{WP}SS$ Dilution 1/16	18	1:4 +	66.0	0.00
$SENASA_{NEG}SS$	NR	NR	3.1	0.00

[a]Standard serum provided by DILAB-SENASA. SP, strong positive; WP, weak positive; NEG, negative; NR, non-reactive. $SENASA_{WP}SS$ dilutions were performed in negative serum.

[b]IU/ml, international complement fixation test units per ml. IU/ml of the dilutions of the standard $SENASA_{WP}SS$ were calculated based on the value reported by SENASA for this serum (288 IU/ml).

[c]CFT results expressed as the highest dilution given a positive result. The plus signs indicate the % of haemolysis inhibition: 25% (+), 50% (++), 75% (+++) and 100% (++++).

[d]VETLIS® *Brucella* Glyco-iELISA Bovine results express as percentage of reactivity. Cut-off values and result interpretation: reactivity >20%, positive; reactivity ≥11% ≤ 20%, indeterminate; reactivity <11%, negative.

[e]Results are expressed as the relative intensity of the test line (TL) with respect to the control line (CL) for each strip. Interpretation of the result: TL/CL = 0, negative; TL/CL >0, positive.

TABLE 3 *Brucella* GlycoStrip analysis of the serum samples included in the double-blind study

		GlycoStrip		
		POS	NEG	
Glyco-iELISA[a]	POS	124	2	126
	IND	1	34	35
	NEG	0	241	241
		125	277	402

[a]VETLIS® *Brucella* Glyco-iELISA Bovine (Chemtest Argentina S. A.). POS, positive result (reactivity >20%); IND, indeterminate (reactivity ≥11% ≤ 20%); NEG, negative result (reactivity <11%).

These results demonstrate that the *Brucella* Glycostrip combines the maximum precision of the Glyco-iELISA with the advantages of a one-step immunochromatographic test which can be performed onsite (POC test) in regions with little or no infrastructure, minimally trained staff and with results in only 10 min.

DISCUSSION

Bovine brucellosis is a worldwide zoonotic disease that causes important economic losses and public health concerns. For these reasons, control programmes have been implemented in many regions and countries, and it is well known that the most cost-effective mechanism to prevent human infections is to control the disease in animal reservoirs. Control and eradication programmes rely on vaccination, serodiagnosis and isolation of the infected animals. Humoral immunity to smooth *Brucella* is dominated by antibodies against the *O*-side chain of the sLPS (Caroff et al., 1984), and the serological tests that allow the differentiation of infected from vaccinated animals are those based on the detection of these types of antibodies (Ciocchini et al., 2014; McGiven et al., 2003; Nielsen, 1990; Nielsen et al., 1995; Nielsen et al., 1996). For these reasons and considering that access to adequate diagnostic tools is a key factor for the implementation of a successful control programme, our goal was to develop a simple and affordable POC/DIVA test for the rapid and accurate diagnosis of bovine brucellosis.

In this work, we have developed and evaluated a novel glycoprotein-based immunochromatographic test for the serodiagnosis of bovine brucellosis. *Brucella* GlycoStrip uses the recombinant glycoprotein OAg-AcrA as an antigen for the detection of specific antibodies against the *O* polysaccharide section of the LPS in serum samples. As we have previously reported, recombinant glycoprotein based-antigens in which the *O* polysaccharide section of the LPS is covalently linked to a carrier protein provides advantages in terms of diagnostic sensitivity and specificity for the serodiagnosis of Gram-negative infectious diseases (Ciocchini et al., 2013; Ciocchini et al., 2014; Cortina, Balzano, et al., 2016; Melli et al., 2015). Furthermore, these antigens are ideal for developing immunochromatographic tests since the carrier protein allows the adsorption of hydrophilic molecules such as the *O* polysaccharides to the hydrophobic nitrocellulose membranes used in lateral flow immunoassays. These characteristics

facilitate the standardization of the dispensing conditions of different recombinant glycoproteins generated by conjugation of different polysaccharides to the same carrier protein. The diagnostic performance of the assay was evaluated using three sample panels. The first panel included pre-infection, post-infection, post-vaccination (with a smooth or rough strains) and post-challenge serum samples obtained from four groups of animals included in a controlled vaccination/challenge trial performed by our laboratory. This analysis allowed us to determine the usefulness of the *Brucella* GlycoStrip for the diagnosis of bovine brucellosis and its capacity to differentiate infected animals from those vaccinated with a smooth or a rough vaccine strain. The second panel included serum samples from naturally infected and non-infected/S19-vaccinated cattle of Argentina. This analysis allowed us to evaluate the performance of the *Brucella* GlycoStrip under the actual epidemiological situation of bovine brucellosis in Argentina and showed a diagnostic sensitivity and specificity of 96.9% and 100%, and a PPV and NPV of 100% and 99.1%, respectively. These results indicate that the *Brucella* GlycoStrip is a highly accurate test for the diagnosis of bovine brucellosis even in countries with eradication campaigns based on the mandatory use of S19. The analysed negative reference samples ($n = 216$) were obtained from non-infected/S19-vaccinated animals including not only BPAT-negative ($n = 159$) but also BPAT-positive samples that were negative by cELISA, FPA and CFT ($n = 57$) and, therefore, considered negative according to the criteria established by the national animal health authority. For BPAT, 26.4% of these samples ($n = 57$) gave a false-positive result probably due to the presence of residual antibodies produced in response to vaccination with S19. Instead, all the negative reference samples were negative when the *Brucella* GlycoStrip was used demonstrating the advantages of this test over the BPAT. Finally, the third panel included reference standard sera generated by the National Brucellosis Reference Laboratory in comparison with the OIE international reference standards and the results demonstrate that the *Brucella* GlycoStrip fulfils the minimum diagnostic requirements accepted and established by the national and international directives.

A further evaluation of *Brucella* GlycoStrip was performed using a double-blind study that included 402 encrypted serum samples obtained from two different livestock farms with a history of brucellosis in Argentina. This analysis allowed us to compare the performance of the *Brucella* GlycoStrip with an ELISA test (VETLIS® *Brucella* Glyco-iELISA Bovine, Chemtest Argentina S. A.) that uses the same antigen (OAg-AcrA) and therefore also detects antibodies against the *O*-side chain of the sLPS. Both tests showed an excellent correlation ($\kappa = 0.987$) demonstrating that the *Brucella* GlycoStrip is as accurate as the Glyco-iELISA with the advantages of being a fast and easy to use POC test that would allow its implementation anywhere, improving diagnostic accessibility. Although several Lateral flow immunoassays (LFIA) for detection of *Brucella* antibodies have been reported (Abdoel et al., 2008; Manasa et al., 2019), they have a lower diagnostic performance than *Brucella* GlycoStrip. These tests use the complete sLPS of *Brucella abortus* as antigen so do not differentiate infected from vaccinated animals and cannot be used in countries where vaccination is mandatory. Furthermore, they may suffer from false-positive results due to cross-reactivity against common epitopes present in the core-lipid A section of sLPS shared by *Brucella* and other Gram-negative bacteria. Instead, the use of the *O*-side chain of *Brucella* LPS as antigen improves the specificity of the test. Recently, a LFIA that uses a double antigen (proteins BP26 and OMP31) sandwich method has been reported (Shi et al., 2022). However, the serological tests based on protein antigens usually have a lower sensitivity in comparison with those that use the *O* polysaccharide as antigen since this molecule is the immunodominant antigen in 'smooth' *Brucella* infections.

According to our results, *Brucella* GlycoStrip is a DIVA test for rough vaccine strains such as *B. abortus* Δ*pgm* (evaluated in this work) and RB51 (not evaluated) because it exclusively detects anti-*O* polysaccharide antibodies, an antigen that is absent in these vaccine strains. When using smooth vaccine strains, although GlycoStrip cannot be considered strictly a DIVA test, it allows to clearly differentiate infected from S19-vaccinated animals when the sampling and analysis is performed from 6 months after vaccination. In the analysis of the serum samples of the animals included in the controlled vaccination/challenge trial, all the samples obtained at 395 days of vaccination with S19 were negative by the GlycoStrip. In addition, in the negative reference panel analysed in this work, the sampling was performed from 6 months after vaccination with a single dose of S19 and of 216 samples, 57 were BPAT positive but negative by the GlycoStrip, cELISA and FPA. These tests (cELISA and FPA) as well as the GlycoStrip specifically and exclusively detect anti-*O* polysaccharide antibodies and are considered among the most suitable tests for assessment of the infection/S19-vaccination status.

In countries with high incidence of bovine brucellosis and where vaccination is mandatory, the combination of sensitive and specific diagnostic assays with a vaccine that does not generate antibody titres that may interfere with the diagnostic methods establishes the principles of a feasible DIVA strategy. Furthermore, the availability of affordable POC tests is critical for the implementation of effective control programmes. In this sense,

the characteristics of the *Brucella* GlycoStrip in terms of speed, simplicity, accuracy, and the possibility of being used as part of a DIVA strategy, in particular for rough vaccine strains or for smooth strains when the samples are obtained from 6 months after vaccination with a single dose, makes it an ideal tool to improve the effectiveness of brucellosis control programmes.

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CONFLICT OF INTEREST

A patent has been filed regarding the diagnostic application of recombinant glycoproteins.

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SUPPORTING INFORMATION

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